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Benzoquinone levels as a function of age and gender of the red flour beetle, *Tribolium castaneum*

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Abstract

Liquid chromatography with both photodiode array and electrochemical detection was used to analyze as a function of age and gender the levels of two ρ-quinones, methyl-1,4-benzoquinone (MBQ) and ethyl-1,4-benzoquinone (EBQ), which are found in defensive secretions of the red flour beetle, *Tribolium castaneum*. We developed a method to simultaneously analyze quinones and hydroquinones excreted from or in homogenates of individual beetles. The major components present in beetle extracts were the benzoquinones and not free or conjugated forms of the hydroquinones. Greater than 95% of the quinone/hydroquinone mixture in extracts was present in the oxidized form. Because of their lability, however, the quinones were quantified indirectly as their hydroquinone derivatives after extraction in dilute acid supplemented with ascorbic acid as a reducing agent. Comparisons of whole body rinses and homogenates revealed that rinses recovered only up to 60% of the total quinones that were extracted after homogenization. The levels recovered also depended on the age and sex of the individual beetles sampled. ρ-Benzoquinones in both male and female beetles increased after adult eclosion and cuticle sclerotization for 40–50 days and then remained at their highest levels (15–21 μg MBQ and 22–32 μg EBQ per beetle) through 80 days posteclosion. Virgin females that were collected 40–80 days after eclosion contained approximately 40% more of these compounds than males of the same age. The build-up of ρ-benzoquinones subsequent to cuticle sclerotization apparently reflects the need for an adequate cuticular barrier for self-protection from these defensive compounds.

Keywords: Quinones; Hydroquinones; Insect; Flour beetle; *Tribolium castaneum*; Defensive secretion; Quantitative analysis; Liquid chromatography; Photodiode array detection; Electrochemical detection

1. Introduction

For tenebrionids and several other types of insects and other invertebrates, ρ-benzoquinones such as methyl-1,4-benzoquinone (MBQ) and ethyl-1,4-benzoquinone (EBQ) are major components of defensive secretions used as repellents and irritants (Schildknecht et al., 1964; Tschinkel, 1975; Blum, 1981; Howard, 1987; Eisner et al., 1998). These compounds are the focus of this investigation because their presence in a commodity such as grain or flour might be used as an indicator of insect infestation.

Benzoquinones are potent electrophiles and much less stable than their reduced hydroquinone forms. Because of the reactivity and toxicity of benzoquinones, precursors that are more stable and less toxic might be expected to be stored in the biosynthetic tissues and storage glands. However, most studies have shown that the quinones themselves are more abundant than the hydroquinones or conjugates thereof (Englehart et al., 1965; Ladisch et al., 1967; Howard, 1987; Pappas and Morrison, 1995).

Several of the previous studies employed analytical methods that required large numbers of insects for sample preparation (Table 1). In addition, beetles of unknown age and sex were selected for analysis, and several different methods were used for quinone extraction. Some reports noted how labile the quinones were

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in both organic and aqueous solutions, even when they were stored at − 20°C in the dark (Alexander and Barton, 1943; Pappas and Morrison, 1995). Gas or liquid chromatography has been used to quantify benzoquinones directly, although the high temperatures required for the former method also may lead to instability (Howard, 1987; Pappas and Wardrop, 1996). An alternative assay was utilized for an indirect measurement of quinone levels in which the quinones were reduced to their more stable hydroquinone derivatives prior to analysis (Alexander and Barton, 1943; Hackman et al., 1948). For a semiquantitative analysis, another method used 2,4-dinitrophenylhydrazine as a derivatizing agent to yield stable hydrazones (Schildknecht et al., 1964).

In many of these studies, rather large variations were observed in the quantities of benzoquinones measured in the insects (Table 1). For example, in *Tribolium* species, levels ranged from undetectable to more than 50 μg per beetle. Therefore, we wanted to develop a more sensitive and precise method of analysis that would be useful for determining the cause of this variation in benzoquinones present in the cosmopolitan stored-product pest, the red flour beetle, *Tribolium castaneum* (Herbst). This report describes a method for quantifying the reduced form of the quinones, the hydroquinones, and also shows that the variation in quinone levels in the insects can be attributed to the labile nature of the quinones as well as both age and gender of the beetles sampled. Extracts of beetles were analyzed using liquid chromatography with photodiode array and electrochemical detection (LC/PDA/EC), which can be used to measure both quinones and hydroquinones simultaneously. This method is arguably one of the most reliable developed to date for quantitative analysis of quinones and hydroquinones.

2. Materials and methods

2.1. Insects

Red flour beetles were maintained on a medium of whole wheat flour plus brewer's yeast (95:5 wt/wt) at 30°C and 57% r.h. To establish sample groups of known age after adult eclosion, pupae were sifted out, sorted into male and female groups after examination of external genitalia, and then held in Petri dishes. Adults that emerged within successive 24 h periods were transferred to plastic culture cups (30 ml). A density of 30 beetles or less per cup was used.

2.2. Chemicals

The following chemicals were obtained from commercial sources and used as received: 1,4-benzoquinone (BQ), 1,4-hydroquinone (HQ), methyl-1,4-benzoquinone (MBQ) and methyl-1,4-hydroquinone (MHQ; Aldrich, Milwaukee, WI); ammonium formate, formic acid, hydrochloric acid and HPLC-grade methanol with a UV cutoff of 204 nm (Fisher Scientific Co., Fairlawn, NJ); and ascorbic acid (Sigma Co., St. Louis, MO). In addition, 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenethylamine (dopamine) were purchased from Sigma. All compounds were stored at −20°C in the dark.

For the stability studies, authentic standards were dissolved in either an aqueous solution of 10% methanol, 10 mM HCl, and 25 mM ascorbic acid (solution A); an aqueous solution containing 1 mM HCl and 1 mM ascorbic acid; an aqueous solution of 10% methanol, 1 mM HCl, and 1 mM ascorbic acid; or 1 mM HCl in 100% methanol. In solutions containing ascorbic acid, BQ and MBQ were reduced to their hydroquinone forms, HQ and MHQ, respectively. To determine the ascorbic acid concentration needed to fully reduce BQ and MBQ, mixtures were prepared in which the molar ratio of ascorbic acid to quinone in 10% methanol plus 10 mM HCl was increased from 0:1 to 35:1 and analyzed by LC/PDA/EC. The ratio that was sufficient for complete reduction of the quinones was approximately 20:1.

2.3. Chromatography

The LC system consisted of a Beckman System Gold gradient liquid chromatography system (Berkeley, CA) equipped with a model 126 solvent module, a model 168 diode array detector, and a Bioanalytical Systems (West Lafayette, IN) LC-4B dual amperometric detector connected via a Beckman interface. All data were collected and processed by Beckman System Gold *Nouveau* software. Separations were achieved using a Phenomenex (Torrance, CA) Prodigy ODS3 PEEK (polyetheretherketone) column (5 μm, 4.6 × 250 mm) equipped with a PEEK guard column. A binary mobile phase consisted of solvent I, 0.15 M formic acid and 0.03 M ammonium formate (pH 3.0), and solvent II, a 1:1 mixture of methanol and a buffer composed of 0.3 M formic acid and 0.06 M ammonium formate. The flow rate was 1 ml/min, and the mobile phase gradients were 0–2 min, 0% solvent II; 2–22 min, 0–100% solvent II; and 22–25 min, 100 to 0% solvent II.

During LC analysis, UV spectra were recorded with the PDA detector using the wavelength range of 190–350 nm. In addition, two fixed wavelength LC/PDA chromatograms, 250 nm for quinone analysis and 290 nm for hydroquinone analysis, were produced for each run. At the same time, chromatograms for electrochemical responses (LC/EC) were obtained for both the oxidation channel A and reduction channel B of the amperometric detector. Potentials applied to the two working electrodes of the amperometric detector were + 800 and −100 mV vs Ag/AgCl for channels A (oxidation) and

Table 1
Analyses of quinones/hydroquinones/catechols in *Tribolium* species

Species	Method	Results	References
T. castaneum	Sublimation of 500–1000 insects per trial with ethanol cold trap and $NaHSO_3$ solvent. Beetle age and sex unknown. Chemical tests and UV spectral analysis.	No quantitative data on quinone amounts. Total secretion was 2.3–4.5 μg/beetle and contained mainly ethylquinones.	Alexander and Barton (1943)
T. castaneum	Sublimation of 25,000–74,000 insects per trial with ether and diethyl ether solvents. Beetle age and sex unknown. Chemical tests.	Total quinones = ~ 16.2 μg/beetle. 10–20% MBQ, 80–90% EBQ, and < 1% 2-methoxy-1,4-benzoquinone.	Loconti and Roth (1953)
4 *Tribolium* spp.	Extraction method not described. Beetle age and sex unknown. Polarographic detection.	MBQ + EBQ = 55.3 ± 14.3 μg/beetle, MHQ + EHQ = 7.0 ± 2.0 μg/beetle, Total Q + HQ = 62.3 ± 13.1 μg/beetle.	Ladisch et al. (1967)
T. castaneum	Defensive secretion and methanolic extracts of secretion cells subjected to TLC. Method of extraction not described. Glands dissected, and chemical tests performed on fixed tissues. Beetle age and sex unknown.	No quantitative data on quinone amounts. Secretion and cellular extract both contained phenolic substances. Migration rate of secretion matched mixture of HQ, MHQ and EHQ. Cellular extracts also contained β-glucoside. Fixed tissue tests indicated presence of phenol glucosides, glucosidases, and oxidases.	Happ (1968)
147 Tenebrionid spp.	Direct gland milking of 1 insect per trial. Carbon disulfide solvent. GC analysis. Beetle age and sex unknown.	No quantitative data on quinone amounts. Secretion of most species contains 40% quinones by weight. MBQ and EBQ were present with latter major. BQ rare.	Tschinkel (1975)
6 *Tribolium* spp.	Cold stress extraction: beetles placed on cold Petri dishes, crystalized secretion collected and mixed with hexane. Beetle age and sex unknown. GC analysis.	Mol% = 37% MBQ, 63% EBQ. Quinones represent 58.3% of secretion by weight.	Markarian et al. (1978)
4 *Tribolium* spp.	Rinses and homogenates of 1–4 insects per trial with hexane/methanol (95:5) solvent. Beetle age and sex unknown. GC analysis.	No quantitative data for *T. castaneum*. Quinones increased until 30–50 days after eclosion. Females contained significantly more quinones than males.	Wirtz et al. (1978)
8 *Tribolium* spp.	Rinses of 1 insect per trial. 2,2,4-trimethylpentane solvent. Beetle age and sex known. GC/MS analysis.	No quantitative data on quinone amounts. Found no taxonomically useful variation in quinones. MBQ, EBQ, MHQ, EHQ found in all species. Up to 11 other compounds detected. No significant difference in secretion components due to gender. Quantity of secretion increased with age.	Howard (1987)
T. castaneum	Homogenates of 6–60 insects per trial with acidic solvents: 1.2 M HCl/0.4 mM sodium metabisulfite and 1 M acetic acid. Beetle age known, sex unknown. LC/EC analysis.	Results for adults ≤ 24 hr, catechol amounts as μg/mg integument: Dopamine = 0.036 ± 0.02, N-acetyldopamine = 0.006 ± 0.002, N-β-alanyldopamine = 0.027 ± 0.004, DOPAC = > 10.1 μg/mg integument.	Roseland et al. (1987)
T. castaneum, T. confusum	Rinses and homogenates of 10–200 insects per trial with methanol solvent. Beetle age and sex unknown. LC/UV/MS analysis.	Surface Q = ~ 0.3 μg/beetle (2.3 nmol), Total Q = ~ 25 μg/beetle (204–209 nmol), Mol% = 38% MBQ, 62% EBQ, MHQ and EHQ detected, but not quantified.	Pappas and Wardrop (1996)
T. castaneum	Homogenates of 1–4 insects per trial with aqueous solvent of 10% methanol, 10 mM HCl, 25 mM ascorbic acid. Beetle age and sex known. LC/UV/EC analysis.	Females at 40 days posteclosion: MBQ + MHQ = 20.4 ± 1.5 μg/beetle, EBQ + EHQ = 32.0 ± 2.7 μg/beetle. Males at 40 days posteclosion: MBQ + MHQ = 15.5 ± 3.0 μg/beetle, EBQ + EHQ = 22.2 ± 4.3 μg/beetle. DOPAC and dopamine also detected.	This study

B (reduction), respectively. The full-scale current of the amperometric detector was set at 500 nA, which was converted to 1 V full-scale output and shown on the LC/EC chromatograms.

2.4. Spectral analyses

Molar absorption coefficients of the authentic standards were determined using the HP 8452A diode array

spectrophotometer and 1 × 0.4 cm quartz cuvettes. The λ_{max} values of BQ and MBQ in a 10% methanol solution were 245 and 250 nm, respectively. The λ_{max} of HQ and MHQ was 290 nm. Quinones and hydroquinones dissolved in an aqueous solution of 10% methanol and 10 mM HCl had the following molar absorption coefficients ($L \cdot cm^{-1} \cdot mol^{-1}$) at 250 nm, $\epsilon_{M,\,BQ} = 18{,}100$ and $\epsilon_{M,\,MBQ} = 16{,}500$; at 290 nm, $\epsilon_{M,\,BHQ} = 2620$ and $\epsilon_{M,\,MHQ} = 2960$.

2.5. Surface quinone analyses

Pappas and Morrison (1995) developed a benzoquinone recovery method in which 50–100 beetles were "washed" in methanol, thereby capturing quinones from the insect surface. In addition, these researchers reported the ability to detect quinones (MBQ and EBQ) in samples of a single beetle rinsed in 0.10 ml methanol using reversed phase LC/UV. Therefore, we attempted to apply the rinsing method of Pappas and Morrison (1995) to samples of 1–10 beetles to compare the effectiveness of various extraction solvents and to determine if the quinone reserve of *T. castaneum* could be exhausted by rinsing alone.

Preliminary studies involved beetles of unknown age and sex placed in test tubes and subjected to a variety of rinsing methods. These involved manipulation of the following: aliquot volume (0.05–1.0 ml), sample size (1–10 beetles), and/or rinsing time (5–30 min). Rinses were carried out at room temperature in 100% methanol with and without 10 mM HCl, and in aqueous solutions containing either methanol (10–50% v/v) or 10 mM HCl. For each trial, the duration of beetle survival in the solutions was noted. Next, three extended-time rinse trials were conducted on samples of four beetles each. Beetles received successive 10 min rinses for either 30 or 90 min and were kept at room temperature (2 trials) or on ice in the dark (1 trial). The rinsing solvent, 10% methanol containing 10 mM HCl and 1 mM ascorbic acid, was removed at the end of each rinsing period. After the final rinse, insects were homogenized in glass tissue grinders in 0.20 ml of fresh solution. Samples were analyzed using LC/PDA/EC within 24 h.

2.6. Total quinone analyses

The primary emphasis of this study was the direct comparison of insect homogenates containing 3–4 beetles of known sex and age (days after adult eclosion) and using a beetle to solution ratio of 1 beetle to 0.15 ml. Each homogenate was centrifuged (15,000***g*** for 5 min) and, after the supernatant was filtered (Millipore 0.45 μm), it was immediately analyzed or stored in glass vials at −20°C in the dark. Samples were analyzed within 48 h. In most trials, solution A was used to reduce the quinones to their hydroquinone forms. In addition, we compared the amounts of hydroquinones extracted from beetle homogenates as outlined above with those extracted in 0.3 M perchloric acid containing 25 mM ascorbic acid. The latter solvent was used routinely to extract phenolic compounds and their conjugates from tissues and to inactivate enzymes.

Another comparison involved duplicate sample aliquots from beetles homogenized in 1.2 M HCl. One aliquot was analyzed immediately while the other was heated for 10 min at 100°C. LC analysis of samples before and after heating in dilute acid provided estimates of the relative amounts of free and conjugated hydroquinones (Hopkins et al., 1995). A final trial compared the total quinones extracted from individual insects homogenized in two different solvents: 10 mM HCl in methanol or solution A. Extraction solvent volume was 0.40 ml per insect. The former solution allowed the direct detection of quinones and hydroquinones, whereas the latter was found to be the most stable environment for the reduced form of the standard compounds. Four sets of chromatograms were obtained from each analysis: absorbance profiles at 250 and 290 nm, one oxidation profile, and one reduction profile.

3. Results

Preliminary analysis of benzoquinones in beetle extracts revealed that the benzoquinones were reduced partially during chromatography by metallic components in the LC system, which also occurred when catecholamine quinones were subjected to a similar procedure (Xu et al., 1995). To avoid these artifacts, a PEEK LC system, which was constructed using an inert organic polymer instead of stainless steel, was utilized for this study.

Initial trials of rinsing the beetle's body surface using 100% methanol followed by LC analysis of aliquots revealed that both quinones and hydroquinones were present in the rinse solution. Improved recovery was obtained by low volume rinses (0.1–0.2 ml) that also contained 10 mM HCl to help stabilize the quinones. In some of the aqueous solutions, however, the insects struggled to stay afloat and continued to secrete defensive material, whereas in the higher percentage methanol solutions, the beetles died upon contact and the quinones recovered were only those already present on the surface at the time of rinsing. Extracts of homogenized beetles collected after the longer duration rinsing trials, however, still contained large quantities of quinones (~ 40–60% of total). Therefore, the focus of the study was shifted to extracts of beetle homogenates.

Stability studies confirmed the results of Pappas and Wardrop (1996) which showed that benzoquinones from whole body homogenates gradually degraded in 100% methanol over several days. In addition, we detected changes in relative amounts of standard quinones and

hydroquinones with time, which indicated that redox cycling reactions occurred in methanol–HCl solutions. Solution A, however, reduced all of the quinones to hydroquinones and afforded a > 95% recovery. Furthermore, stability tests showed that benzoquinones when reduced to hydroquinones remained stable in that solution for at least two weeks, when stored in the dark at ≤ -20°C. Standard curves of LC response (peak area) vs concentration were developed for BQ and MBQ dissolved in methanol containing 10 mM HCl, as well as for BHQ and MHQ dissolved in solution A. Because authentic standards of EBQ and EHQ were not available commercially, the MBQ and MHQ standard curves were used to estimate concentrations of EBQ and EHQ in insect samples.

Fig. 1 illustrates the retention times of hydroquinones and catechols during LC and compares their contents in homogenates and body rinse samples. Although older beetles (80 days posteclosion) were used for this analysis, the resulting EC and PDA chromatograms are, nonetheless, characteristic for beetles at any age. In the first subset of chromatograms (A and B), the sample injected was obtained from a non-diluted homogenate (0.6 ml) of four 80-day old males. The high concentrations in that sample and the high sensitivity of the EC detector led to oxidative peaks that exceeded the 1 V full scale setting, whereas PDA responses at 290 nm were on scale. In the second subset (C and D), the sample was from four 80-day old females rinsed for 1 h in 0.6 ml of solution and then diluted to 2 ml. Diluting the sample yielded EC peak responses that were on scale but the PDA peaks were small and there was a baseline shift due to an increasing amount of methanol in the gradient.

Although this study used homogenates of three to four beetles for the age and gender analyses, the sensitivity of our method allows the detection of hydroquinones and quinones from much less than one beetle equivalent. For example, with an extract of four beetles in 0.6 ml solution and an injection volume of 20 μl, approximately 0.13 beetle equivalent was analyzed per injection. Furthermore, with extracts of one beetle in 0.4 ml solution and an injection volume of 20 μl, only 0.05 beetle equivalent was analyzed. In trials involving authentic standards, we determined that ng amounts of MHQ could be detected by EC (oxidation channel), whereas UV (@290 nm) detection required approximately 20-fold more of the compound. The sensitivity of UV analysis, however, is four times greater for quinones than for hydroquinones. UV detection at 250 nm required as little as 12 ng MBQ or 50 ng MHQ. On the other hand, EC peak area response in our system for the hydroquinone was about two times greater than that for an equivalent amount of quinone.

In the EC and PDA chromatograms obtained from insect extracts [Fig. 1(A–D)], MBQ and MHQ were readily identified because their UV spectra and retention times (22.3 and 18.1 min, respectively) were identical to those of the authentic standards. Another compound that eluted more than 7 min after MBQ was identified as EBQ, because its UV absorbance spectrum was typical for a quinone (λ_{max} = 250 nm) and also because other researchers have reported EBQ as a major component in *Tribolium* extracts (Loconti and Roth, 1953; Tschinkel, 1975; Markarian et al., 1978; Howard, 1987; Pappas and Wardrop, 1996). Likewise, a compound that eluted more than 6 min later than MHQ was identified as EHQ based upon spectral analysis (λ_{max} = 290 nm) and prior reports in the literature of EHQ being present in *Tribolium* extracts (Englehart et al., 1965; Howard, 1987; Pappas and Wardrop, 1996). In 100% methanol extracts, the quinone to hydroquinone molar ratio was approximately 97:3 for both MBQ:MHQ and EBQ:EHQ. Therefore, the major components of these redox couples found in the beetles in vivo were the oxidized forms.

Besides MHQ and EHQ, both PDA and EC chromatograms for beetles of ages less than 10 days after eclosion also exhibited substantial amounts of two other electroactive compounds with retention times of 8.4 and 18.4 min (Fig. 1). These compounds were identified as the catechols, dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC), respectively, based on comparable UV absorbance spectra and retention times of standard compounds. Dopamine exhibited a maximum level at day 2 post-eclosion, whereas DOPAC showed a maximum concentration at day 5 (data not shown). Levels of both of these catechols fell by more than 50% by day 10 and decreased even more thereafter. These data for the levels of the two catechols were consistent with those of Kramer et al. (1984) and Roseland et al. (1987), who demonstrated an early build up of both dopamine and DOPAC in *T. castaneum* after adult eclosion, followed by declines. Because body surface rinses contained only the 1,4-benzoquinones and not the catechols, the latter apparently were not part of the defensive secretion but were putative metabolites utilized for adult cuticle tanning (Hopkins and Kramer, 1992).

Previously, studies have indicated that benzoquinones produced by *Tribolium*, because of their high toxicity, may be stored in the defensive glands primarily in the form of hydroquinone glucosides. These conjugates then are hydrolyzed by a glucosidase, and the resulting hydroquinones are oxidized enzymatically to benzoquinones as they exit the glands (Eisner et al., 1964; Happ, 1968; Pappas and Morrison, 1995). We investigated this possibility by determining the levels of benzoquinones and hydroquinones before and after heating extracts of beetle homogenates in dilute acid. This treatment hydrolyzes glycosidic bonds of hydroquinone conjugates. The recovery of hydroquinones under these conditions was 85 $\pm$ 6% (n = 3). Surprisingly, the major effect of the heating was not an increase in hydroquinone content but, instead, a large decrease in benzoquinone content. More

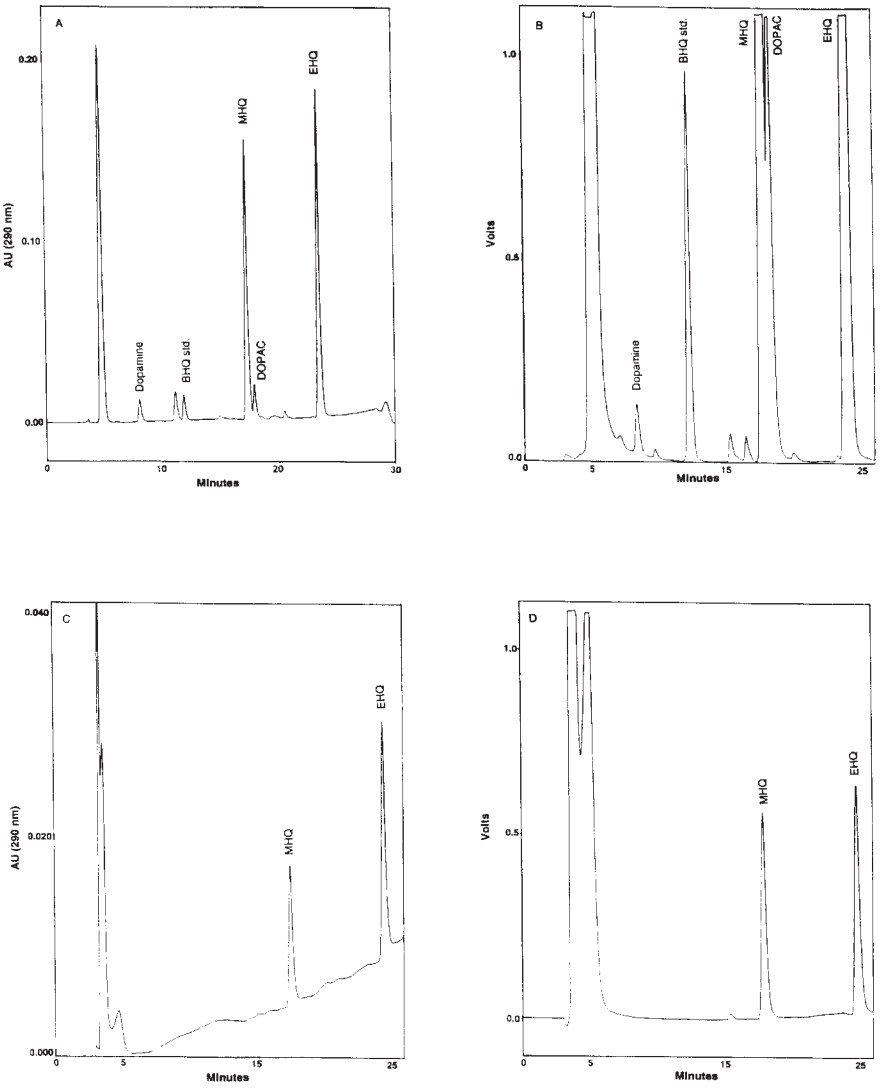

Fig. 1. HPLC of quinones, hydroquinones, and catechols in *Tribolium castaneum* homogenate extracts (A and B) and body rinses (C and D). Four male red flour beetles, 80 days after eclosion, were homogenized in 0.60 ml of 10% methanol, 10 mM HCl, 25 mM ascorbic acid. Twenty microliters of the supernatant were injected. (A) LC/PDA (290 nm) chromatogram. Peaks were identified as follows: ascorbic acid, 4.7 min; dopamine, 8.1 min; BHQ internal standard, 12.0 min; MHQ, 17.5 min; DOPAC, 18.2 min and EHQ, 23.6 min. (B) LC/EC (oxidation) chromatogram. Peaks were identified as follows: ascorbic acid, 5.0 min; dopamine, 8.4 min; BHQ internal standard, 12.3 min; MHQ, 18.1 min; DOPAC, 18.4 min. and EHQ, 24.0 min. Four female red flour beetles, 80 days after eclosion, were rinsed for 1 h in 0.6 ml of 10% methanol, 10 mM HCl, 25 mM ascorbic acid, which was diluted to 2.0 ml. Two hundred microliters were injected. (C) LC/PDA (290 nm) chromatogram. Peaks were identified as follows: ascorbic acid, 4.7 min.; MHQ, 17.6 min and EHQ, 24.5 min. (D) LC/EC (oxidation) chromatogram. Peaks were identified as follows: ascorbic acid, 5.0 min.; MHQ, 18.1 min and EHQ, 25.0 min.

than 90% of the benzoquinones decomposed, whereas only a 1.4 to 2.1-fold increase occurred in the hydroquinones, which was minor relative to the quinones present in unheated extracts. Thus, these data indicated that only a relatively small portion of the gland contents ($<$ 20%) is present as either free or conjugated hydroquinones and that $>$ 80% is benzoquinones.

Concentrations of quinones as a function of time after adult eclosion were determined by LC/PDA/EC and are reported as μg per insect in Fig. 2. Each data point is the mean value obtained from five to eight replicates of extracts of three to four insects each. In extracts of newly eclosed adults, levels of MBQ and EBQ were very low, $<$ 0.1 and $<$ 0.3 μg per insect, respectively, and were revealed by EC detection only. However, both of the quinones increased with time until approximately 40–50 days posteclosion. The average total concentration of all quinones at 40 days posteclosion was approximately 45 μg per insect, with levels of 18 μg MBQ and 27 μg EBQ per insect. At day 80, total quinone concentrations were not significantly different from the overall total for day 40 beetles. Virgin females contained higher levels of MBQ than males at 20 and 40 days after eclosion, whereas EBQ levels in females were significantly higher

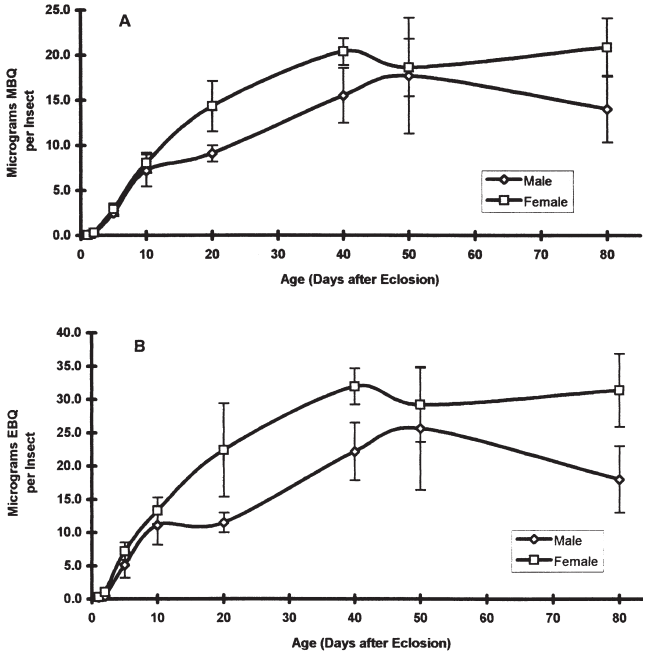

Fig. 2. Benzoquinone levels of adult *Tribolium castaneum* (micrograms per beetle) as a function of gender and days after adult eclosion. Data are from 3–4 beetles homogenized in aqueous 10% methanol, 10 mM HCl and 25 mM ascorbic acid (0.15 ml per beetle). (A) Methyl-1,4-benzoquinone. (B) Ethyl-1,4-benzoquinone.

than those in males at 20, 40, and 80 days after eclosion. Day 40 females contained about 20 μg MBQ per insect compared to 16 μg per insect for males. EBQ levels at that time were 32 μg per female and 22 μg per male. However, day 40 males and females showed similar MBQ mol% values of 44% and 42%, respectively. Thus, not only age but also gender can influence the benzoquinone levels in red flour beetles.

4. Discussion

Researchers have investigated the defensive secretions of a variety of tenebrionid beetles, in particular *Tribolium* species, which infest stored products and contaminate them with their defensive secretions and fecal materials (Schildknecht et al., 1964; Tschinkel, 1975; Blum, 1981; Omaye et al., 1981; Howard, 1987; El-Mofty et al., 1989). The quinone content of these secretions has been a focus of study, because these electrophiles constitute a major portion of the secretion and their detection in a commodity might be an indicator of insect infestation. Both chemical and chromatographic methods of analyses have been used. For example, Tschinkel (1975) used gas chromatography to analyze extracts of secretory glands from over 100 tenebrionid species and found that quinones accounted for > 40% of the composition, with EBQ predominating over MBQ in most species and benzoquinone rarely occurring at all (Table 1). MBQ is a major constituent of secretions of all diplopods and in tenebrionids, where it is almost always accompanied by EBQ (Blum, 1981). Furthermore, another study found that the quinones represented more than half of the *T. castaneum* secretions by weight (Markarian et al., 1978). In addition to the expected variation among tenebrionid species, Tschinkel (1975) reported a high degree of variation in the quinone concentrations among individuals of the same species. In Table 1, we compared the results of our study of quinone concentrations of *T. castaneum* to those reported by other laboratories. All of the amounts are reported as μg per insect to make a direct comparison.

Although several studies have focused on the isolation and characterization of *Tribolium* beetle secretions, few have attempted to determine quinone concentrations for individual adults (Ladisch et al., 1967; Wirtz et al., 1978; Howard, 1987; Pappas and Morrison, 1995). Some of the early studies of the defensive secretions of red flour beetles involved rather large sample sizes and tedious extraction methods. One method involved the gaseous capture of secretions from large sample groups (1000 to 74,000 insects) through sublimation (40°C) for 6–24 h with cold trap collection. Using this method, Alexander and Barton (1943) first reported that the secretion of *T. castaneum* contained "mainly ethylquinones", whereas Loconti and Roth (1953) found quinone concentrations of ~ 16 μg per insect (30 g per 1.85 million insects) as well as relative molar percentages of 10–20% MBQ, 80–90% EBQ, and trace amounts of 2-methoxy-1,4-benzoquinone. In contrast, Markarian et al. (1978) used a gland milking technique and found 37% MBQ and 63% EHQ in the secretion of *T. castaneum*. The former value is very close to the value of 38% MBQ present in methanolic whole-body rinses and homogenates obtained by Pappas and Wardrop (1996). We obtained slightly higher mol% values of 41–44% MBQ and 56–59% EBQ. Our finding of 45–49 μg total quinones per insect was slightly lower than the 50–76 μg per insect range of quinone concentrations found in *T. castaneum* by Ladisch et al. (1967), who used a polarographic detection method. In comparison, the 16 μg and 25 μg total quinones per insect reported by Loconti and Roth (1953) and Pappas and Wardrop (1996) were considerably lower. However, the data obtained in those studies represented mean values for adults of mixed ages and sex. Not only can age and gender influence the levels of defensive compounds in *Tribolium*, but also ecological factors such as availability of food, photoperiod, and beetle density and strain, as well as health. Some strains of *T. castaneum* exhibited up to a threefold increase in benzoquinones after infection by the rat tapeworm, *Hymenolepsis diminuta*, for which the flour beetle is an intermediate host (Yan and Phillips, 1996).

We also found that *T. castaneum* beetles contained very low levels of benzoquinones immediately after

eclosion, but the quinones increased from day 1 to 40 after eclosion. Beetles at 40–80 days post eclosion showed little change in total quinone levels. Concentrations were significantly higher in females than males for EBQ at 20, 40, and 80 days after eclosion and for MBQ at 20 and 40 days. Females in our cultures were isolated from males, and because we did not sample the insects prior to homogenization, the defensive material was allowed to accumulate over time. These findings agree with trends reported by Wirtz et al. (1978), who investigated in two other *Tribolium* species, *T. brevicornis* and *T. madens*, the effects of age and gender on quinone levels and described a similar build up of quinones after adult eclosion. Those authors referred to unpublished data indicating that female *T. castaneum* and *T. confusum* adults contained significantly higher quinone concentrations than males of the same age. Furthermore, Roth (1943) observed an increase in the volume of secretory material stored in the abdominal reservoirs of *T. confusum* as the beetles aged. Howard (1987) also reported age-related changes in the quantity of secretion produced by *Tribolium* species but noted that the chemical composition of the secretion was constant over time and between males and females. The opposite trend was reported for *Zophobas atratus*, a tenebrionid species whose males produced more quinones than the females (Hill and Tschinkel, 1985). Those authors also noted that mated *Zophobas* females exhibited a lower secretion recharge rate than virgin females.

Studies by Eisner et al. (1964); Hurst et al. (1964), and Happ (1968) indicated that quinone production within the secretory cells of *T. castaneum* and *Eleodes longicollis* proceeds from hydroquinone glucosides to free hydroquinones and finally to the oxidized benzoquinones. Presumably, the secretory cells found in the linings of the glandular sacs avoid self-poisoning by producing the less reactive hydroquinones and their conjugates within the cell cytoplasm and then transferring them to a cuticular vesicular organelle within the cell where the final synthesis of the quinones occurs. In *Tribolium* the benzoquinones then flow through cuticular tubes into a cuticle-lined storage sac, where large reserves of the secretion build up and remain stable (Roth, 1943; Happ, 1968). In addition, Howard (1987) identified up to 11 other secretory components in eight *Tribolium* species, of which unsaturated hydrocarbons were major. A hydrophobic environment such as in these sacs may help to stabilize and disperse the quinones when secreted. Interestingly, Schildknecht et al. (1964) found measurable quantities of quinones in the abdominal sacs of 100-year-old museum specimens of tenebrionids. In our study, we determined that the amounts of hydroquinones in the red flour beetle liberated from conjugates by heating in dilute acid represented $< 6\%$ of the total benzoquinone–hydroquinone mixture. The amounts of conjugates present were relatively low, and the benzoquinones themselves were the principal storage compounds.

Tschinkel (1975) reported that although all tenebrionids manufacture benzoquinones, only the most primitive species produce quinones without any hydrocarbon components, which, in the morphologically advanced tenebrionids, serve as additional irritants as well as spreading agents (Peschke and Eisner, 1987). In *Blaps mucronata*, the quinone/hydrocarbon mixture first disperses along grooves in the elytra and then eventually over the beetles' entire surface area (Peschke and Eisner, 1987). Obviously, tenebrionids are somewhat protected from their own secretions both internally and externally by cuticular linings, and methods of self-protection must have co-evolved with the ability to synthesize secretions. Within *Tribolium* species, this self-protection is evident in the partitioning of the secretion away from cells, first in the cuticle-lined organelles where the secretion is produced (Happ, 1968) and then in storage sacs that are formed from invaginations of the cuticle (Roth, 1943). We propose that the lack of defensive secretion in newly emerged *Tribolium* adults reflects the need for an adequate barrier for self-protection. Adult cuticle sclerotization in *T. castaneum* requires approximately 4 days for completion (Roseland et al., 1987). Only after that time does a rapid build-up of benzoquinones occur in adults (Wirtz et al., 1978; this study). In addition, the metabolic pathways for synthesizing the benzoquinones in defensive secretions and catecholamine quinones used for cuticle tanning may share some of the same precursors, and in young adults, the benzoquinone biosynthetic pathway may be suppressed in favor of the catecholamine quinone pathway until cuticle tanning agents are no longer needed. Roseland et al. (1987) did not quantify catechols in older red flour beetles (> 20 days posteclosion) but did report that beetles at 7 days posteclosion still contained DOPAC at a level of approximately 20 nmoles per mg elytra, whereas dopamine levels were more than 100-fold lower. In this study, we found detectable levels of catechols in whole body homogenates from beetles as old as 80 days posteclosion (Fig. 1) but observed a decline in concentration over time in contrast to the build up in benzoquinones.

In conclusion, with the highly sensitive PEEK LC/PDA/EC system, we were able to determine simultaneously the benzoquinone and hydroquinone levels in individual flour beetles and to demonstrate that their levels depend on the age and gender of individuals. We also found that benzoquinones are the major components present in the beetles and not the hydroquinones or conjugates thereof. Many invertebrates use these electrophiles as repellents and irritants towards natural enemies. The occurrence of these and other electroactive compounds in defensive secretions of individuals from other species of insects will be the subject of future studies. In addition, the potential for using this analytical system

for detecting *Tribolium* species in wheat flour will be evaluated. Hodges et al. (1995) did not find substantial accumulations of quinones from flour beetles in dehusked rice, but contamination of wheat flour appears to be a more serious problem. Rheological properties of the dough are highly sensitive to electroactive compounds such as quinones and exposure of flour to moderate numbers of beetles for only a few weeks is sufficient to reduce its rheological properties (Payne, 1925).

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References

Alexander, P., Barton, H.R., 1943. The excretion of ethylquinone by the flour beetle. Biochem. J. 37, 463–465.

Blum, M.S., 1981. Chemical Defenses of Arthropods. Academic Press, New York, pp. 183–205.

Eisner, T., McHenry, F., Salpeter, M.M., 1964. Defensive mechanisms of arthropods. J. Morphol. 115, 355–399.

Eisner, T., Eisner, M., Attygalle, A.B., Deyrup, M., Meinwald, J., 1998. Rendering the inedible edible: circumvention of a millipede's chemical defense by a predaceous beetle larva (Phengodidae). Proc. Natl. Acad. Sci. USA 95, 1108–1113.

El-Mofty, M.M., Sakr, S.A., Osman, S.I., Toulan, B.A., 1989. Carcinogenic effect of biscuits made with flour infested with *Tribolium castaneum* in *Bufo regularis*. Oncology 46, 63–65.

Englehart, M., Rapoport, H., Sokoloff, A., 1965. Odorous secretion of normal and mutant *Tribolium confusum*. Science 150, 632–633.

Hackman, R.H., Pryor, M.G.M., Todd, A.R., 1948. The occurrence of phenolic substances in arthropods. Biochem. J. 43, 474–477.

Happ, G.M., 1968. Quinone and hydrocarbon production in the defensive glands of *Eleodes longicollis* and *Tribolium castaneum* (Coleoptera, Tenebrionidae). J. Insect Physiol. 14, 1821–1837.

Hill, C.S., Tschinkel, W.R., 1985. Defensive secretion production in the tenebrionid beetle *Zophobus atratus*: effects of age, sex and milking frequency. J. Chem. Ecol. 11, 1083–1092.

Hodges, R.J., Robinson, R., Hall, D.R., 1995. Quinone contamination of dehusked rice by *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). J. Stored Prod. Res. 32, 31–37.

Hopkins, T.L., Kramer, K.J., 1992. Insect cuticle sclerotization. Annu. Rev. Entomol. 37, 273–302.

Hopkins, T.L., Morgan, T.D., Mueller, D.D., Tomer, K.B., Kramer, K.J., 1995. Identification of catecholamine β-glucosides in the hemolymph of the tobacco hornworm, *Manduca sexta* (L.), during development. Insect Biochem. Molec. Biol. 25, 29–37.

Howard, R.W., 1987. Chemosystematic studies of the Triboliini (Coleoptera: Tenebrionidae): phylogenetic inferences from the defensive chemicals of eight *Tribolium* spp., *Palorus ratzeburgi* (Wissmann), and *Latheticus oryzae* Waterhouse. Ann. Ent. Soc. Amer. 80, 398–405.

Hurst, J.J., Meinwald, J., Eisner, T., 1964. Defense mechanisms of arthropods—XII. Glucose and hydrocarbons in the quinone-containing secretion of *Eleodes longicollis*. Ann. Ent. Soc. Amer. 57, 44–46.

Kramer, K.J., Morgan, T.D., Hopkins, T.L., Roseland, C.R., Aso, Y., Beeman, R.W., Lookhart, G.L., 1984. Catecholamines and β-alanine in the red flour beetle, *Tribolium castaneum*. Roles in cuticle sclerotization and melanization. Insect Biochem. 14, 293–298.

Ladisch, R.K., Ladisch, S.K., Howe, P.M., 1967. Quinoid secretions in grain and flour beetles. Nature 215, 939–940.

Loconti, J.D., Roth, L.M., 1953. Composition of the odorous secretion of *Tribolium castaneum*. Ann. Ent. Soc. Amer. 46, 281–289.

Markarian, H., Florentine, G.J., Pratt, J.J., 1978. Quinone production of some species of *Tribolium*. J. Insect Physiol. 24, 785–790.

Omaye, S.T., Wirtz, R.A., Fruin, J.T., 1981. Toxicity of substituted ρ-benzoquinones found in the secretion of tenebrionid flour beetles. Proc. West. Pharmacol. Soc. 24, 169–171.

Pappas, P.W., Morrison, S.E., 1995. Benzoquinones of the beetles *Tribolium castaneum* and *Tribolium confusum*. Prep. Biochem. 25, 155–168.

Pappas, P.W., Wardrop, S.M., 1996. Quantification of benzoquinones in the flour beetles, *Tribolium castaneum* and *Tribolium confusum*. Prep. Biochem. Biotech. 26, 53–66.

Payne, N., 1925. Some effects of *Tribolium* on flour. J. Econ. Entomol. 18, 737–743.

Peschke, K., Eisner, T., 1987. Defensive secretion of the tenebrionid beetle, *Blaps mucronata*: physical and chemical determinants of effectiveness. J. Comp. Physiol. A161, 377–388.

Roseland, C., Kramer, K.J., Hopkins, T.L., 1987. Cuticular strength and pigmentation of rust-red and black strains of *Tribolium castaneum*: correlation with catecholamine and β-alanine content. Insect Biochem. 17, 21–28.

Roth, L.M., 1943. Studies on the gaseous secretion of *Tribolium confusum* Duval. Ann. Ent. Soc. Amer. 36, 397–425.

Schildknecht, H., Holoubek, K., Weis, K.H., Krämer, H., 1964. Defensive substances of arthropods, their isolation and identification. Angew. Chemie Internat. Ed. 3, 73–156.

Tschinkel, W.R., 1975. A comparative study of the chemical defensive system of tenebrionid beetles: Chemistry of the secretions. J. Insect Physiol. 21, 753–783.

Wirtz, R.A., Taylor, S.L., Semey, H.G., 1978. Concentrations of substituted ρ-benzoquinones and 1-pentadecene in the flour beetles *Tribolium madens* (Charp.) and *T. brevicornis* (Lec.) (Coleoptera, Tenebrionidae). Comp. Biochem. Physiol. 61C, 287–290.

Xu, R., Huang, X., Kramer, K.J., Hawley, M.D., 1995. On-column reduction of catecholamine quinones in stainless steel columns during liquid chromatography. Anal. Biochem. 231, 72–81.

Yan, G., Phillips, T.W., 1996. Influence of tapeworm infection on the production of aggregation pheromone and defensive compounds in *Tribolium castaneum*. J. Parasitol. 82, 1037–1039.